Chapter 3
Analytical Methods

3.2.4 ESTIMATION OF PHOSPHATIDYL CHOLINE IN LIPOSOMES

The Stewart assay (Stewart, 1980) was used for estimating phosphatidyl choline in liposomes. This method utilizes the ability of phospholipids to form a complex with ammonium ferrothiocyanate in organic solution.

3.2.4.1 Solutions

1. Ammonium ferrothiocyanate solution (0.1M) was prepared by dissolving 27.03g of ferric chloride hexahydrate and 30.4g of ammonium thiocyanate in double distilled water and making up the volume of the resulting solution to 1 litre.
2. Stock solution of phosphatidyl choline in chloroform (0.1mg/ml) was prepared by dissolving 50mg of phosphatidyl choline in 10ml of chloroform. 2ml of this solution was diluted 100 times to yield a solution of the required concentration.
3. Saturated sodium chloride solution: Sodium chloride was dissolved in distilled water with heating to form a supersaturated solution. This solution was then cooled to room temperature and filtered to give the required saturated solution.

3.2.4.2 Procedure for calibration curve

Suitable aliquots (0.1 –1.5 ml) of the stock solution of phosphatidyl choline were transferred to 10ml centrifuge tubes. Appropriate quantities of chloroform were then added such that the total volume of the contents of the tubes was 3ml. To each tube, 2ml of ammonium ferrothiocyanate solution (0.1M) was then added. The contents of each tube were mixed by vigorous vortexing on a cyclomixer for 15sec. The tubes were then spun for 5min at 1800 rpm in a tabletop centrifuge. The lower, organic colored layer was then removed using a syringe and long needle (18 gauge) and transferred to a test tube. The absorbance of these solutions was measured at 485 nm on a Shimadzu 1601 UV-Visible spectrophotometer with glass cells of 10mm path length using a blank prepared in the same manner omitting the phospholipids.

The above procedure was repeated six times. Mean absorbance values along with the regressed values (method of least squares) and the regression equation obtained are shown in Table 3.1. The optical characteristics of the colored solutions are tabled in Table 3.2.

Table 3.1 Calibration curve for estimation of phosphatidyl choline (Stewart assay)

Concentration (μg/3ml)	Mean Absorbance* ± S.E.	Regressed value
10	0.0296 ± 0.0014	0.0305
20	0.0549 ± 0.0025	0.0587
40	0.1206 ± 0.0033	0.1150
60	0.1741 ± 0.0019	0.1713
80	0.2244 ± 0.0026	0.2276
100	0.2833 ± 0.0018	0.2839

Regression equation** Y= 0.0028 X + 0.0024
Correlation coefficient = 0.9986
*Mean of 6 values
**n=36.

Table 3.2 Optical characteristics for ammonium ferrothiocyanate-phosphatidyl choline complex in chloroform

Characteristic	Value
Absorption maxima (nm)	485
Beer's law limits (μg/3ml)	10-100
Apparent molar absorptivity (l/mol/cm)	6651.7
Sandell's sensitivity coefficient (S) ($\mu g/cm^2$/0.001 abs unit)	1.1×10^{-4}

3.2.4.3 Stability and selectivity

Stability of the colored solutions, prepared above for the calibration curve of phosphatidyl choline, was ascertained by observing the changes in absorbance of the solutions over a period of 4 h. The selectivity of the method for phosphatidyl choline was investigated by carrying out the procedure detailed above in the presence of potential interferences such as cholesterol, methotrexate, tamoxifen citrate, cytarabine, etc., at the levels at which these materials were included in the liposomes.

3.2.4.4 Estimation of phosphatidyl choline from liposomes/ supernatant

The Bligh-Dyer two-phase extraction method (New, 1990) was modified for estimating phosphatidyl choline from liposomes. Briefly, 0.1ml of liposomal dispersion or 0.5ml of the supernatant was taken in a centrifuge tube and to this 1ml of saturated sodium chloride solution was added. 2ml of chloroform was then added to the contents followed by vigorous vortexing on a cyclomixer for 30 sec and centrifugation at 1800 rpm for 5 min in a tabletop centrifuge. The lower chloroform layer was separated using a syringe and needle (18 gauge) and passed over a bed of anhydrous sodium sulphate into a 10ml volumetric flask. The process was repeated with a further 2ml and 1ml of chloroform. The chloroform layers were then pooled and the volume made up to 10ml with chloroform. To 0.5ml of this chloroform extract in a centrifuge tube, 2.5ml of chloroform and 2ml of 0.1M ammonium ferrothiocyanate solution was added. The contents were then subjected to the same procedure as detailed above for the standards (Section 3.2.4.2). Duplicate estimations were performed and the mean absorbance was used to determine the amount of phosphatidyl choline in the liposomes or supernatant using the regression equation.

3.2.5 ESTIMATION OF CHOLESTEROL IN LIPOSOMES

The Zlatkis, Zak and Boyle's method was used for estimating cholesterol in liposomes. This method utilizes the ability of cholesterol in acetic acid to form a complex with ferric chloride and sulphuric acid (Zlatkis *et. al.,* 1953).

3.2.5.1 Solutions

1. Ferric chloride solution: A 0.05% w/v solution of ferric chloride hexahydrate in glacial acetic acid was prepared by dissolving 50mg of ferric chloride hexahydrate in 100ml glacial acetic acid.
2. Stock solution of cholesterol: A 0.5mg/ml solution of cholesterol in glacial acetic acid was prepared by dissolving 25mg of cholesterol in 50ml of glacial acetic acid.
3. Saturated sodium chloride solution: It was prepared in the same manner as for the estimation of phosphatidyl choline from liposomes (Section 3.2.4.1)

3.2.5.2 Procedure for calibration curve

Suitable aliquots of the stock solution of cholesterol (0.1 to 1ml) were transferred accurately into 10ml volumetric flasks. To each flask, 4ml of ferric chloride solution and 4ml of concentrated sulphuric acid was added. The contents were mixed, made up to the volume with glacial acetic acid and allowed to stand for 30 min. The absorbance of the resulting colored solutions of the complex, formed between cholesterol, ferric chloride and sulphuric acid was measured at 550nm using Shimadzu 1601 UV-Visible spectrophotometer with glass cells of 10mm path length, against a blank prepared in the same manner as the standard solutions except cholesterol.

The above procedure was repeated six times. The experimental data along with the results of the statistical evaluation of the data are shown in Table 3.3. The optical characteristics for the above solutions of the complex formed are tabled in Table 3.4.

3.2.5.3 Stability and selectivity

Stability of the solutions of the complex, prepared for obtaining the calibration curve of cholesterol, was ascertained by observing the changes in their absorbances over a period of 24 h.

Cholesterol was estimated in the presence of phosphatidyl choline, methotrexate, tamoxifen citrate, cytarabine, etc., at the same concentrations at which these materials were included in the liposomes to ascertain the selectivity of the method.

3.2.5.4 Estimation of cholesterol from liposomes/ supernatant

1ml of the chloroform extract, obtained as described before in the estimation of phosphatidyl choline form liposomes or supernatant (Section 3.2.4.4) was taken in a 10ml volumetric flask and evaporated to dryness by heating at 90°C in a thermostatically controlled, electrically heated water bath. The dried contents were then subjected to the same procedure as discussed previously (Section 3.2.5.2). The amount of cholesterol in the liposomes or supernatant was then obtained using the regression equation of the calibration curve.

Table 3.3 Calibration curve for estimation of cholesterol (Zlatkis, Zak and Boyle's method)

Concentration (μg/ml)	Mean Absorbance* ± S.E.	Regressed value
5	0.067 ± 0.0024	0.071
10	0.155 ± 0.0019	0.157
20	0.343 ± 0.0019	0.328
30	0.478 ± 0.0012	0.499
40	0.696 ± 0.0018	0.671
50	0.832 ± 0.0017	0.843

Regression equation** Y= 0.0172X –0.0146

Correlation coefficient = 0.9968

*Mean of 6 values

**n=36.

Table 3.4 Optical characteristics for cholesterol -ferric chloride-sulphuric acid complex in glacial acetic acid

Characteristic	Value
Absorption maxima (nm)	550
Beer's law limits (μg/ml)	5-50
Apparent molar absorptivity (l/mol/cm)	6007.8
Sandell's sensitivity coefficient (S) ($\mu g/cm^2$/0.001 abs unit)	6.4×10^{-5}

3.2.6 ESTIMATION OF METHOTREXATE IN LIPOSOMES

Methotrexate in methanol shows strong absorbance in the ultraviolet region of the electromagnetic spectrum (Arthur *et.al.,* 1976).

3.2.6.1 Solutions

Stock solution of methotrexate (100μg/ml) was prepared by dissolving 10mg of methotrexate in 100ml of methanol.

3.2.6.2 Procedure for calibration curve

Suitable aliquots (0.5 –2.5ml) of the stock solution of methotrexate were pipetted into 10ml volumetric flasks and the volume was made up to 10ml with methanol to give final concentrations of 5, 10, 15, 20 and 25μg/ml. The solutions were shaken well and their absorbances measured at 303nm using methanol as blank on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated six times. Table 3.5 tabulated the raw and regressed data (method of least squares) so obtained whereas Table 3.6 contains the optical characteristics for the solution of methotrexate in methanol. Absorptivity scan over the UV wavelength range between 200 and 330 nm for 20μg/ml solution of methotrexate in methanol is shown in Figure 3.1.

3.2.6.3 Stability and selectivity

Stability of the solutions of methotrexate in methanol, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 24h at room temperature.

The above method for estimating methotrexate was carried out in the presence of phosphatidyl choline, cholesterol, α-tocopherol and other components of the liposomes to ascertain the selectivity of the method.

3.2.6.4 Estimation of methotrexate from liposomes/ supernatant

To 0.1ml of liposomes or 0.5ml of supernatant in a 10ml volumetric flask, methanol was added to break the liposomes and the volume was made up with methanol. The absorbance was measured at 303nm against a blank comprising of empty liposomes diluted with methanol in the similar manner. Triplicate estimations were made and the mean absorbances were determined. The amount of methotrexate in the liposomes or supernatant was then obtained using the regression equation.

Table 3.5 Calibration curve for methotrexate in methanol

Concentration (µg/ ml)	Mean Absorbance* ± S.E.	Regressed Value
5	0.292 ± 0.0023	0.284
10	0.606 ± 0.0022	0.611
15	0.939 ± 0.0017	0.938
20	1.245 ± 0.0018	1.265
25	1.607 ± 0.0016	1.592

Regression equation[++]: Y = 0.0654X - 0.0654

Correlation co efficient = 0.9993

*Mean of 6 values

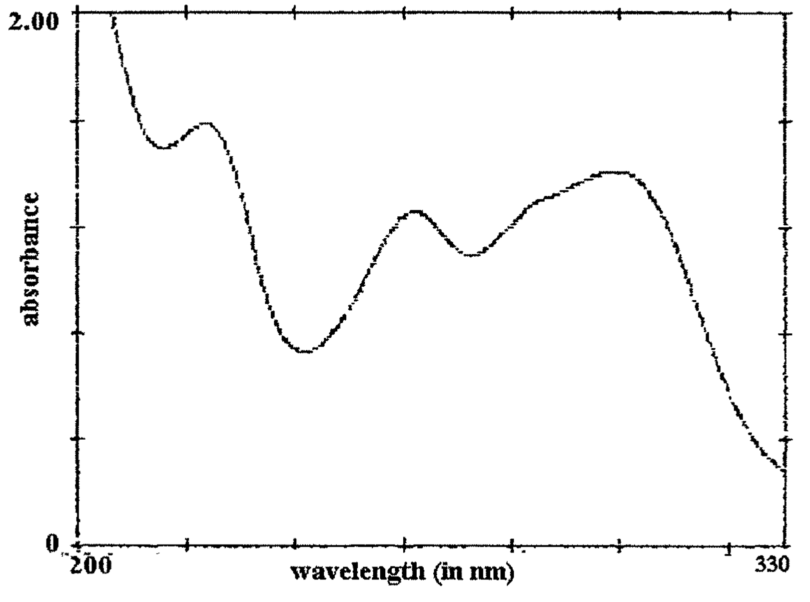
[++]n=30

Table 3.6 Optical characteristics for methotrexate in methanol

Characteristic	Value
Absorption maxima (nm)	222, 257, 303*
Beer's law limits (µg/ml)	5-25
Apparent molar absorptivity (l/mol/cm)	27545.4
Sandell's sensitivity coefficient (S) (µg/cm²/0.001 abs unit)	1.6×10^{-5}

* Analytical wavelength

Figure 3.1 Absorptivity scan of methotrexate in methanol (20µg/ml)



3.2.7 ESTIMATION OF TAMOXIFEN CITRATE IN LIPOSOMES

Tamoxifen citrate in methanol shows strong absorbance in the ultraviolet region of the electromagnetic spectrum.

3.2.7.1 Solutions

Stock solution of tamoxifen citrate (100μg/ml) was prepared by dissolving 10mg of tamoxifen citrate in 100ml of methanol.

3.2.7.2 Procedure for calibration curve

Suitable aliquots (0.5 –5ml) of the stock solution of tamoxifen citrate were pipetted into 10ml volumetric flasks and the volume was made up to 10ml with methanol to give final concentrations of 5, 10, 20, 30, 40 and 50μg/ml. The solutions were shaken well and their absorbances measured at 274nm using methanol as blank on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated six times. Table 3.7 tabulated the raw and regressed data (method of least squares) so obtained whereas Table 3.8 contains the optical characteristics for the solution of tamoxifen citrate in methanol. Absorptivity scan over the UV wavelength range between 200 and 330nm for 30μg/ml solution of tamoxifen citrate in methanol is shown in Figure 3.2.

3.2.7.3 Stability and selectivity

Stability of the solutions of tamoxifen citrate in methanol, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 24h at room temperature.

The above method for estimating tamoxifen citrate was carried out in the presence of phosphatidyl choline, cholesterol, α-tocopherol and .other components of the liposomes to ascertain the selectivity of the method.

3.2.7.4 Estimation of tamoxifen citrate from liposomes/ supernatant

To 0.1ml of liposomes or 0.5ml of supernatant in a 10ml volumetric flask, methanol was added to break the liposomes and the volume was made up with methanol. The absorbance was measured at 274nm against a blank comprising of empty liposomes diluted with methanol in the similar manner. Triplicate estimations were made and the mean absorbances were determined. The amount of tamoxifen citrate in the liposomes or supernatant was then obtained using the regression equation.

Table 3.7 Calibration curve for tamoxifen citrate in methanol

Concentration (µg/ ml)	Mean Absorbance* ± S.E.	Regressed Value
5	0.112 ± 0.0051	0.113
10	0.217 ± 0.0080	0.218
20	0.429 ± 0.0099	0.429
30	0.653 ± 0.0108	0.639
40	0.831 ± 0.0094	0.850
50	1.067 ± 0.0089	1.060

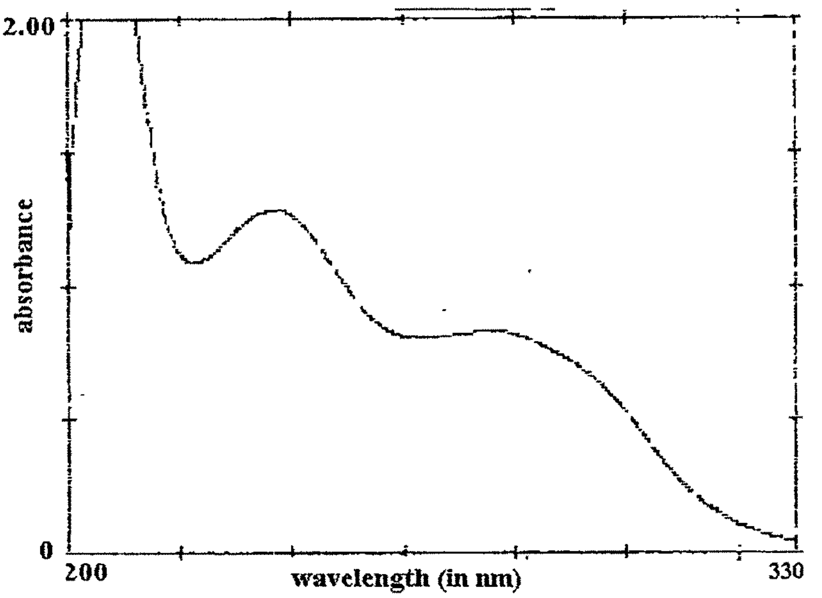
Regression equation^{++}: Y = 0.02X + 0.008
Correlation co efficient = 0.9991
*Mean of 6 values
$^{++}$n=36

Table 3.8 Optical characteristics for tamoxifen citrate in methanol

Characteristic	Value
Absorption maxima (nm)	212, 236, 274*
Beer's law limits (µg/ml)	5-50
Apparent molar absorptivity (l/mol/cm)	13070
Sandell's sensitivity coefficient (S) (µg/cm²/0.001 abs unit)	4.3 x 10^{-5}

*Analytical wavelength

Figure 3.2 Absorptivity scan of tamoxifen citrate in methanol (30μg/ml)



3.2.8 ESTIMATION OF CYTARABINE IN LIPOSOMES

This method was developed based on the observation that cytarabine in methanol shows strong absorbance in the ultraviolet region of the electromagnetic spectrum.

3.2.8.1 Solutions

Stock solution of cytarabine (100μg/ml) was prepared by dissolving 10mg of cytarabine in 100ml of methanol.

3.2.8.2 Procedure for calibration curve

Suitable aliquots (0.5 – 5ml) of the stock solution of cytarabine were pipetted into 10ml volumetric flasks and the volume was made up to 10ml with methanol to give final concentrations of 5, 10, 20, 30, 40 and 50μg/ml. The solutions were shaken well and their absorbances measured at 274nm using methanol as blank on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated six times. Table 3.9 tabulated the raw and regressed data (method of least squares) so obtained whereas Table 3.10 contains the optical characteristics for the solution of cytarabine in methanol. Absorptivity scan over the UV wavelength range between 200 and 400nm for 25μg/ml solution of cytarabine in methanol is shown in Figure 3.3.

3.2.8.3 Stability and selectivity

Stability of the solutions of cytarabine in methanol, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 24h at room temperature.

The above method for estimating cytarabine was carried out in the presence of phosphatidyl choline, cholesterol, α-tocopherol and other components of the liposomes to ascertain the selectivity of the method.

3.2.8.4 Precision and accuracy

In order to determine precision and accuracy of the method, solutions containing known amounts of cytarabine were prepared and analysed using the procedure detailed above, in three replicates. The analytical results obtained from these investigations for both methods are summarized in Table 3.11.

3.2.8.5 Estimation of cytarabine from liposomes/ supernatant

To 0.1ml of liposomes or 0.5ml of supernatant in a 10ml volumetric flask, methanol was added to break the liposomes and the volume was made up with methanol. The absorbance was measured at 274nm against a blank comprising of empty liposomes diluted with methanol in the similar manner. Triplicate estimations were made and the mean absorbances were determined. The amount of cytarabine in the liposomes or supernatant was then obtained using the regression equation.

Table 3.9 Calibration curve for cytarabine in methanol

Concentration (µg/ ml)	Mean Absorbance* ± S.E.	Regressed Value
5	0.202 ± 0.0110	0.193
10	0.356 ± 0.0110	0.371
20	0.724 ± 0.0093	0.726
30	1.086 ± 0.0100	1.081
40	1.454 ± 0.0100	1.437
50	1.779 ± 0.0076	1.792

Regression equation++: Y = 0.0355X + 0.015

Correlation co efficient = 0.9998

*Mean of 6 values

++n=36

Table 3.10 Optical characteristics for cytarabine in methanol

Characteristic	Value
Absorption maxima (nm)	209, 274*
Beer's law limits (µg/ml)	5 –50
Apparent molar absorptivity (l/mol/cm)	8829
Sandell's sensitivity coefficient (S) (µg/cm²/0.001 abs unit)	2.75×10^{-5}

*Analytical wavelength

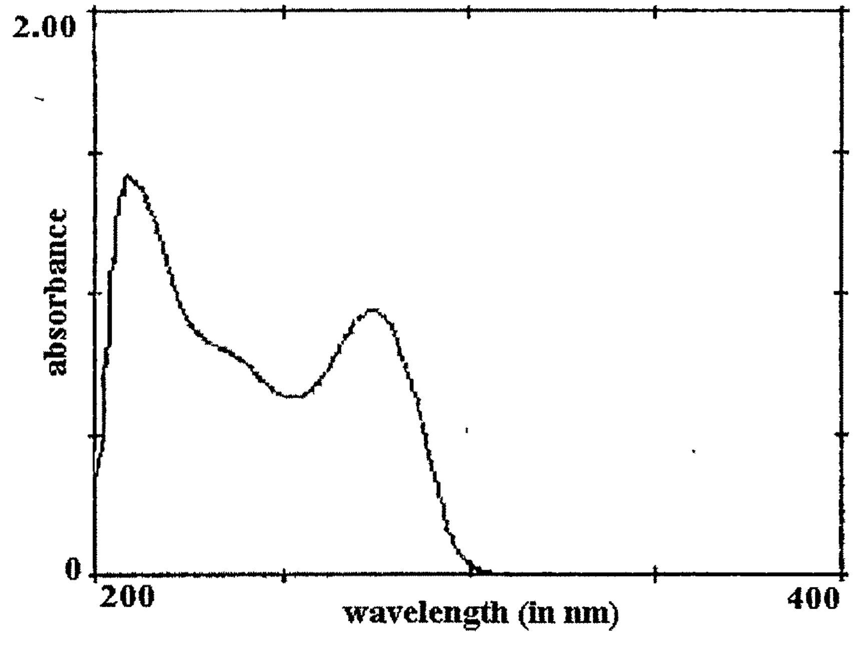
Table 3.11 Evaluation of accuracy and precision of the method for estimation of cytarabine in methanol

Exact amount of drug added (mg)	Individual amounts found (mg) ± (S.D.)[a]	Coefficient of variation (CV)	Relative mean error (RME)	Confidence limits[b]
25	24.97 ± 0.321	0.0128	0.0039	24.97 ± 1.27
50	49.87 ± 0.473	0.0095	0.0033	49.87 ± 1.58
100	99.9 ± 0.436	0.0044	0.0023	99.9 ± 1.08

[a] n = 3.

[b] Confidence limits at P = 0.95 and two degree of freedom.

Figure 3.3 Absorptivity scan of cytarabine in methanol (25μg/ml)



3.2.9 ESTIMATION OF METHOTREXATE IN PHOSPHATE BUFFERED SALINE pH 7.4 (PBS)

Methotrexate in phosphate buffered saline pH 7.4 (PBS) shows strong absorbance in the ultraviolet region of the electromagnetic spectrum (Arthur *et. al.,* 1976).

3.2.9.1 Solutions

1. Phosphate buffered saline pH 7.4 (PBS) was prepared as per the procedure given in the Indian Pharmacopoeia (1985).
2. Stock solution of methotrexate (100μg/ ml) was prepared by dissolving 10mg of methotrexate in 100ml of methanol.

3.2.9.2 Procedure for calibration curve

Suitable aliquots (0.5 –2.5ml) of the stock solution of methotrexate were pipetted into 10ml volumetric flasks and the volume was made up to 10ml with PBS to give final concentrations of 5, 10, 15, 20 and 25μg/ml. The solutions were shaken well and their absorbances measured at 303nm using PBS as blank on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated six times. Table 3.12 tabulated the raw and regressed data (method of least squares) so obtained whereas Table 3.13 contains the optical characteristics for the solution of methotrexate in PBS. Absorptivity scan over the UV wavelength range between 200 and 330 nm for 20μg/ml solution of methotrexate in PBS is shown in Figure 3.4.

3.2.9.3 Stability and selectivity

Changes in the absorbance of the solutions of methotrexate in PBS, used for preparing the calibration curve, at the analytical wavelength over a period of 24h at room temperature was used as a mean to study the stability of these solutions with respect to time.

The above method for estimating methotrexate was carried out in the presence of phosphatidyl choline, cholesterol, α-tocopherol and other components of the liposomes to ascertain the selectivity of the method.

Table 3.12 Calibration curve for methotrexate in PBS

Concentration (μg/ ml)	Mean Absorbance* ± S.E.	Regressed Value
5	0.290 ± 0.0023	0.286
10	0.554 ± 0.0018	0.544
15	0.796 ± 0.0031	0.803
20	1.028 ± 0.0014	1.061
25	1.345 ± 0.0024	1.319

Regression equation^{++}: Y = 0.0517X + 0.0274

Correlation co efficient = 0.9972

*Mean of 6 values

$^{++}$n=30

Table 3.13 Optical characteristics for methotrexate in PBS

Characteristic	Value
Absorption maxima (nm)	303
Beer's law limits (μg/ml)	5-25
Apparent molar absorptivity (l/mol/cm)	25182
Sandell's sensitivity coefficient (S) ($\mu g/cm^2$/0.001 abs unit)	1.8×10^{-5}

Figure 3.4 Absorptivity scan of methotrexate in PBS (20µg/ml)

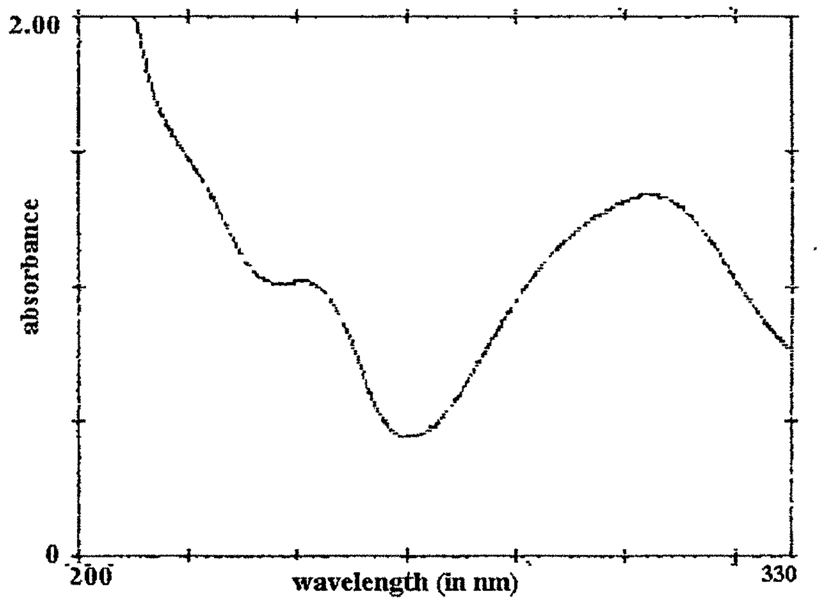


Figure 3.5 Absorptivity scan of tamoxifen citrate in PBS (40µg/ml)

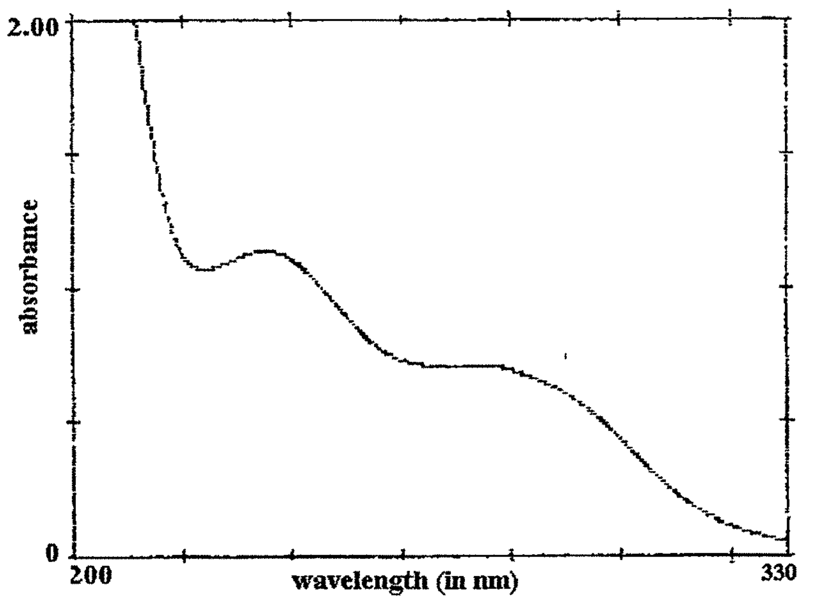


Table 3.14 Calibration curve for tamoxifen citrate in PBS

Concentration (µg/ ml)	Mean Absorbance* ± S.E.	Regressed Value
5	0.086 ± 0.0018	0.083
10	0.161 ± 0.0026	0.165
20	0.338 ± 0.0014	0.330
30	0.487 ± 0.0032	0.495
40	0.653 ± 0.0021	0.658
50	0.832 ± 0.0017	0.825

Regression equation++: Y = 0.0165x + 0.0002

Correlation co efficient = 0.9994

*Mean of 6 values

++n=36

Table 3.15 Optical characteristics for tamoxifen citrate in PBS

Characteristic	Value
Absorption maxima (nm)	236, 274*
Beer's law limits (µg/ml)	5-50
Apparent molar absorptivity (l/mol/cm)	9096
Sandell's sensitivity coefficient (S) (µg/cm^2/0.001 abs unit)	6.2 x 10^{-5}

*Analytical wavelength

3.2.11 ESTIMATION OF CYTARABINE IN PHOSPHATE BUFFERED SALINE pH 7.4 (PBS)

This method was developed based on the observation that cytarabine in PBS shows strong absorbance in the ultraviolet region of the electromagnetic spectrum.

3.2.11.1 Solutions

Stock solution of cytarabine (100μg/ml) was prepared by dissolving 10mg of cytarabine in 100ml of PBS.

3.2.11.2 Procedure for calibration curve

Suitable aliquots (0.5 – 5ml) of the stock solution of cytarabine were pipetted into 10ml volumetric flasks and the volume was made up to 10ml with PBS to give final concentrations of 5, 10, 20, 30, 40 and 50μg/ml. The solutions were shaken well and their absorbances measured at 272nm using PBS as blank. The above procedure was repeated six times. Table 3.16 tabulated the raw and regressed data (method of least squares) so obtained whereas Table 3.17 contains the optical characteristics for the solution of cytarabine in PBS. Absorptivity scan over the UV wavelength range between 200 and 400nm for 25μg/ml solution of cytarabine in PBS is shown in Figure 3.6.

3.2.11.3 Stability and selectivity

Stability of the solutions of cytarabine in PBS, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 24h at room temperature.

The above method for estimating cytarabine was carried out in the presence of phosphatidyl choline, cholesterol, α-tocopherol and other components of the liposomes to ascertain the selectivity of the method.

3.2.11.4 Precision and accuracy

In order to determine precision and accuracy of the method, solutions containing known amounts of cytarabine were prepared and analysed using the procedure detailed above, in three replicates. The analytical results obtained from these investigations for both methods are summarized in Table 3.18.

Table 3.16 Calibration curve for cytarabine in PBS

Concentration (µg/ ml)	**Mean Absorbance* ± S.E.**	**Regressed Value**
5	0.189 ± 0.0095	0.194
10	0.379 ± 0.0071	0.392
20	0.813 ± 0.0054	0.787
30	1.191 ± 0.0068	1.182
40	1.553 ± 0.0081	1.577
50	1.978 ± 0.0048	1.972

Regression equation^{++}: Y = 0.0395x - 0.0034
Correlation co efficient = 0.9997
*Mean of 6 values
$^{++}$n=36

Table 3.17 Optical characteristics for cytarabine in PBS

Characteristic	**Value**
Absorption maxima (nm)	272
Beer's law limits (µg/ml)	5 –50
Apparent molar absorptivity (l/mol/cm)	9683
Sandell's sensitivity coefficient (S) (µg/cm^2/0.001 abs unit)	2.5 x 10^{-5}

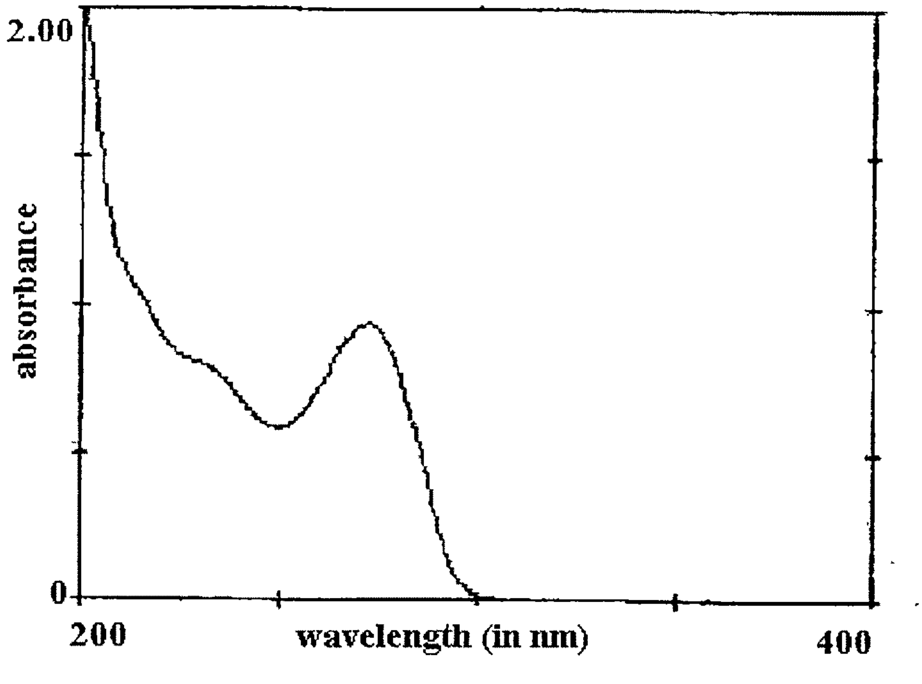
Table 3.18 Evaluation of accuracy and precision of the method for estimation of cytarabine in PBS

Exact amount of drug added (mg)	Individual amounts found (mg) ± (S.D.)[a]	Coefficient of variation (CV)	Relative mean error (RME)	Confidence limits[b]
25	25.07 ± 0.153	0.0061	0.0026	25.07 ± 0.38
50	49.80 ± 0.265	0.0053	0.0022	49.8 ± 0.66
100	99.93 ± 0.379	0.0038	0.0010	99.93 ± 0.94

[a] n = 3.

[b] Confidence limits at P = 0.95 and two degree of freedom.

Figure 3.6 Absorptivity scan of cytarabine in PBS (25μg/ml)



3.2.12 ESTIMATION OF POLYETHYLENE GLYCOL DERIVATIVES IN LIPOSOMES

The estimation of polyethylene glycol derivatives [methoxy polyethylene glycol 5000 activated with cyanuric chloride-phosphatidylethanolamine conjugate (mPEG5000-CC-PE), methoxy polyethylene glycol 2000 activated with cyanuric chloride-phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE) and methoxy polyethylene glycol 5000 activated with imidazoline carbonyl -phosphatidylethanolamine conjugate (mPEG5000-IC-PE)] were done using the method reported by Shimada *et. al.,* (2000).

This method is based on the spectrophotometric determination of complexes of polyethylene glycols with sodium ions after their extraction as picrates, into 1,2-dichloromethane, and provided a sensitive tool for determination of PEG-lipids.

3.2.12.1 Solutions

To a solution containing 0.1M sodium hydroxide, 280g of sodium nitrate (3.3 mol) and 4.58g of picric acid (0.02M) were added and the final volume of the solution was adjusted to 1000 ml. After sonication, the solution was filtered off and stored in a refrigerator at 5°C.

Stock solution of polyethylene glycol derivatives (mPEG5000-CC-PE/ mPEG2000-CC-PE/ mPEG5000-IC-PE) (1mg/ml) was prepared by dissolving 100mg of polyethylene glycol (mPEG5000-CC-PE/ mPEG2000-CC-PE/ mPEG5000-IC-PE) in 100ml of distilled water.

3.2.12.2 Procedure for calibration curve

Suitable aliquots (0.5 – 5ml) of the stock solution of polyethylene glycol derivatives were pipetted into 5ml volumetric flasks and the volume was made up to 5ml with distilled water to give final concentrations of 10, 20, 40, 60, 80 and 100μg/ml. To the 5ml of above sample solution, 10ml of sodium nitrate picrate solution and 5ml of dichloromethane was added. The solution was shaken well and the absorbance was measured at 378nm on a Shimadzu 1601 UV-Visible spectrophotometer. The above procedure was repeated six times. Tables 3.19, 3.21 and 3.23 tabulated the raw and regressed data (method of least squares) so obtained whereas Tables 3.20, 3.22 and 3.24 contains the optical characteristics for the solutions of polyethylene glycol derivatives (mPEG5000-CC-PE/mPEG2000-CC-PE/mPEG5000-IC-PE) in dichloromethane.

3.2.12.3 Stability and selectivity

Stability of the solutions of polyethylene glycol derivatives, used for preparing the calibration curve, was ascertained by observing the changes in their absorbance at the analytical wavelength, over a period of 24h at room temperature.

The above method for estimating polyethylene glycol derivatives was carried out in the presence of methotrexate, tamoxifen citrate, cytarabine, phosphatidyl choline, cholesterol, α-tocopherol and other components of the liposomes to ascertain the selectivity of the method.

3.2.12.4 Estimation of polyethylene glycol derivatives from liposomes/ supernatant

0.1ml of liposomal suspension or 0.5ml of supernatant was diluted to 5ml with distilled water and was mixed with 10ml of sodium nitrate-picrate solution. To the mixture, 5ml of 1, 2-dichloromethane was added. After vigorous shaking, the solution was centrifuged at 1500rpm for 10min and the organic layer was collected and spectrophotometrically measured at 378nm.

Table 3.19 Calibration curve for mPEG5000-CC-PE

Concentration (μg/ ml)	Mean Absorbance* ± S.E.	Regressed Value
10	0.137 ± 0.011	0.161
20	0.303 ± 0.016	0.288
40	0.552 ± 0.010	0.540
60	0.81 ± 0.024	0.793
80	1.024 ± 0.017	1.046
100	1.301 ± 0.019	1.299

Regression equation^{++}: Y = 0.013X + 0.035

Correlation coefficient = 0.9982

*Mean of 6 values

$^{++}$n=36

Table 3.20 Optical characteristics for mPEG5000-CC-PE

Characteristic	Value
Absorption maxima (nm)	378
Beer's law limits (μg/ml)	10-100
Apparent molar absorptivity (l/mol/cm)	75639
Sandell's sensitivity coefficient (S) ($\mu g/cm^2$/0.001 abs unit)	7.6×10^{-5}

Table 3.21 Calibration curve for mPEG2000-CC-PE

Concentration (μg/ ml)	Mean Absorbance* ± S.E.	Regressed Value
10	0.112 ± 0.0026	0.118
20	0.233 ± 0.0034	0.233
40	0.478 ± 0.0061	0.462
60	0.691 ± 0.0045	0.691
80	0.897 ± 0.017	0.920
100	1.162 ± 0.011	1.149

Regression equation++: Y = 0.0115X + 0.0034

Correlation coefficient = 0.9988

*Mean of 6 values

++n=36

Table 3.22 Optical characteristics for mPEG2000-CC-PE

Characteristic	Value
Absorption maxima (nm)	378
Beer's law limits (μg/ml)	10-100
Apparent molar absorptivity (l/mol/cm)	32677
Sandell's sensitivity coefficient (S) (μg/cm^2/0.001 abs unit)	8.6 x 10^{-5}

Table 3.23 Calibration curve for mPEG5000-IC-PE

Concentration ($\mu g/ ml$)	Mean Absorbance* ± S.E.	Regressed Value
10	0.142 ± 0.0026	0.167
20	0.326 ± 0.0064	0.298
40	0.556 ± 0.012	0.559
60	0.834 ± 0.0094	0.821
80	1.064 ± 0.016	1.082
100	1.349 ± 0.018	1.344

Regression equation^{++}: Y = 0.0131X + 0.036
Correlation coefficient = 0.9981
*Mean of 6 values
$^{++}$n=36

Table 3.24 Optical characteristics for mPEG5000-IC-PE

Characteristic	Value
Absorption maxima (nm)	378
Beer's law limits ($\mu g/ml$)	10-100
Apparent molar absorptivity (l/mol/cm)	79352
Sandell's sensitivity coefficient (S) ($\mu g/cm^2/0.001$ abs unit)	7.36×10^{-5}

3.3 RESULTS AND DISCUSSION

3.3.1 ESTIMATION OF PHOSPHATIDYL CHOLINE IN LIPOSOMES

The Stewart assay was used for estimating phosphatidyl choline in liposomes (Stewart, 1980). This method is based on complex formation between ammonium ferrothiocyanate and phospholipids in organic solution. The complex in chloroform exhibits maximum absorbance at 485nm. The advantage of this method is that the presence of inorganic phosphate does not interfere with the assay. A disadvantage, however, is that this method is not applicable to samples where mixtures of unknown phospholipids may be present (New, 1990a).

Absorptivity of the complex was calculated as 6651.7 l/mol/cm (Table 3.2). A correlation coefficient of 0.9986 (Table 3.1) indicated a linear relationship between absorbance and concentration of phosphatidyl choline taken for complex formation. Beer's law was found to be obeyed between 10 – 100 μg/3ml (Table 3.2). This high range is a reflection of the low absorptivity of the complex at the analytical wavelength. The data of regression analysis on the collected data along with the raw data are presented in Table 3.1. The regression equation obtained was $Y = 0.0028X + 0.0024$

The variance of the response variable, S^2_{yx} was obtained as 1.63×10^{-5}. This low value denoted the closeness of experimental points to the least squares line and thus, the low variability in the procedure. This low variability can also be seen from the low value of the standard error of the mean absorbances of the solutions used for obtaining the calibration curve (Table 3.1). The variance of the slope, S^2_b was calculated as 2.68×10^{-9}. The value of the slope (0.0028, Table 3.1) indicated the sensitivity of the method, a fact supported by the high value of the Sandell's sensitivity coefficient (1.1×10^{-4} μg/cm²/0.001 abs unit, Table 3.2). These observations can be attributed to the low absorptivity of the complex (6651.7 l/mol/cm) as already indicated before.

The variance of the intercept, S^2_a, calculated was 9.88×10^{-6}. The significance of the intercept was examined using the null hypothesis. The value of 't' was obtained as 0.7635 whereas the value of 't' required for significance is 2.78 at 4 degrees of freedom at the 5% level. Thus, the null hypothesis can be accepted which means that the blank does not interfere in the absorbance measurements. The stability of the complex was ascertained by measurement of absorbances of the solutions, used for preparing the calibration curve, at regular intervals of time. It was observed that the colour of the complex was retained at its original intensity for an hour. Consequently, it is recommended that the absorbances be measured within an hour of sample preparation and this precaution was followed in all studies involved in this method.

The selectivity of the method for phosphatidyl choline was established by estimating phosphatidyl choline in the presence of the other major constituents of the liposomes viz. cholesterol, methotrexate, tamoxifen citrate and cytarabine at the levels at which these materials were included in the liposomes. None of the materials interfered in any way in the estimation of phosphatidyl choline when the Stewart assay was used for the purpose of estimation.

Being a widely accepted and used method no need was felt to establish accuracy and precision of the method.

The method was used for estimating phosphatidyl choline from liposomes and from the supernatant obtained in the centrifugation step used for removing the unentrapped drug. A modified version of the Bligh-Dyer two-phase extraction method (New, 1990) was used alone instead of chloroform: methanol mixture, recommended in the reported method, as it was found to give results comparable to these obtained with the original method. Also traces of methanol are reported to interfere with the final partition step in the estimation procedure (Stewart, 1980). As chloroform was liable to form an emulsion with phosphate buffered saline, saturated with sodium chloride was added to prevent this. The chloroform layers were passed through a bed of anhydrous sodium sulphate to remove any traces of water. The chloroform extract so obtained was then made up to 10ml and a portion subjected to the procedure detailed above for the estimation of phosphatidyl choline. Mass balance studies revealed good correlation between the amount of phosphatidyl choline added and the amounts recovered from the liposomes and supernatant. Thus the method was found to be satisfactory for estimation of phosphatidyl choline in liposomes.

3.3.2 ESTIMATION OF CHOLESTEROL IN LIPOSOMES

The Zlatkis, Zak and Boyle method (Zlatkis *et. al.,* 1953) was used for estimating cholesterol in liposomes. Here, cholesterol in acetic acid forms a colored complex with ferric chloride and sulphuric acid, which exhibits maximum absorbance at 560nm.

Absorptivity of the complex calculated was 6007.8 (Table 3.4). Linearity of the method was observed from a correlation coefficient of 0.9968 (Table 3.3). Beer's law was found to be obeyed between 5 –50 μg/ml (Table 3.4). Regression analysis of the experimental data was performed and is tabulated along with the experimental data in Table 3.3. The regression equation obtained was Y = 0.0172 X + 0.0146.

The variance of the response variable, S^2_{yx} was calculated as 3.56 x 10^{-4}. This low value illustrated the good fit between the determined and calculated data and thus indicated the low variability of the experiments. This low variability is also reflected in the low value of the standard error of the mean absorbances of the solutions used for obtaining the calibration curve. The variance of the slope, S^2_b was calculated as 2.34 x 10^{-7}. The value of the slope (0.0172, Table 3.3) indicates moderate sensitivity of the method. This observation is strengthened by the low values of the absorptivity of the complex mentioned before, the wide range of concentration in which Beer's law is obeyed and the high value of the Sandell's sensitivity coefficient (6.4 x 10^{-5} μg/cm^2/0.001 abs unit, Table 3.4). The variance of the intercept, S^2_a, was calculated as 2.15 x 10^{-4}. From the null hypothesis testing, it was seen that the blank does not absorb at the wavelength of interest (calculated 't' = 0.9957, tabulated 't' = 2.78 at 4 degrees of freedom, at 5% level).

Monitoring of the absorbances of the solutions, used for preparing the calibration curve revealed that the color of the complex was retained as its original intensity for 24h. ANOVA studies of the mean absorbance values of the solutions at different times revealed no significant difference between the readings. Thus, the complex is stable over a period of 24h.

The presence of the other constituents of the liposomes such as phosphatidyl choline, methotrexate, tamoxifen citrate and cytarabine at the levels at which these materials were included in the liposomes, did not interfere with the estimation of cholesterol.

Being widely accepted and used method, no need was felt to establish accuracy and precision of the method. Finally the method was used to estimate cholesterol in liposomes and supernatant when the liposomes were centrifuged to remove unentrapped drug. The modified Bligh-Dyer two phase extraction method, described earlier (section 3.3.1) was used to extract cholesterol from liposomes. A portion of the chloroform extract, obtained by following the procedure detailed in the aforementioned section, was evaporated to dryness by heating on a water bath at 90°C. Care was taken that no chloroform splashed out of the volumetric flask during the process. The contents of the flask were then dissolved in glacial acetic acid before they were subjected to the procedure detailed earlier for the estimation of the cholesterol. There was good agreement between the amount of cholesterol estimated from liposomes and supernatant and that added initially when liposomes were being prepared, indicating the suitability of the method for the estimation of cholesterol from these types of preparations.

Certain precautions need to be taken when the method is being used. If the solutions are shaken prior to measuring the absorbance, upon transferring to cuvettes, air bubbles are seen in the cuvette impending absorbance measurements. The solutions, therefore, need to be transferred carefully to the cuvette, only when they are devoid of air bubbles and that too with no turbulence. Also, the reagents used in the method (concentrated sulphuric acid and glacial acetic acid) are quite corrosive and should be handled carefully.

3.3.3 ESTIMATION OF METHOTREXATE IN LIPOSOMES

Methotrexate in methanol yields a characteristic curve when scanned in the U.V. wavelength range between 200 to 330nm. The scan (Figure 3.1) shows absorption maxima at 222, 257 and 303nm (Table 3.6). Though the absorptivity of the compound was high at 222nm, it was not selected as the analytical wavelength due to its proximity to the lower limit of the UV transparent region of methanol (≈220nm). The absorptivity at 303nm

(27545 l/mol/cm, Table 3.6) was also found to be satisfactory and hence was selected as the analytical wavelength and used for further investigations. The regression equation was found to be Y =0.0654X - 0.0429.

A correlation coefficient of 0.9993 (Table 3.5) indicated that absorbance and concentration of the drug were linearly related. Beer's law was found to be obeyed between 5 and 25 μg/ml (Table 3.6). Experimental and calculated values for the method are presented in Table 3.5. The slope of the regressed line (0.0654, Table 3.5) indicates moderate sensitivity of the method as also seen by the value of the Sandell's sensitivity coefficient (1.6×10^{-5}), which, once again, reflects the low absorptivity of the compound.

There was a little variability between the experimental and regressed values. This conclusion was based on the low value of the variance of the response variable, S^2_{yx} (2.38×10^{-4}) and the low values of the standard error of the mean absorbances of the solutions used for preparing the calibration curve. The variance of the slope, S^2_b, was calculated as 9.52×10^{-7} whereas the intercept S^2_a, was determined to be 2.93×10^{-4}. The blank (methanol) does not interfere in the measurements as concluded from t' test of the intercept (calculated t' = 2.51, tabulated t' = 3.18 at 3 degrees of freedom, at 5% level).

The mean absorbance values of the methanolic solutions of methotrexate at different concentrations at preselected time intervals was determined, ANOVA studies of the results indicated no significant difference between the readings. Thus, methotrexate is stable over a period of 24h in methanol.

The presence of the other constituents of the liposomes such as phosphatidyl choline and cholesterol at the levels at which these materials were included in the liposomes, did not interfere with the estimation of methotrexate.

The method was used to estimate the amount of methotrexate entrapped in liposomes and its concentration in the supernatant, obtained during the recovery of the liposomes by centrifugation. Good mass balance was obtained between the amount of methotrexate added and that recovered from the liposomes and supernatant, signifying the suitability of the method for this application.

3.3.4 ESTIMATION OF TAMOXIFEN CITRATE IN LIPOSOMES

Tamoxifen citrate in methanol yields a characteristic curve when scanned in the U.V. wavelength range between 200 to 330nm. The scan (Figure 3.2) shows absorption maxima at 212, 236 and 274nm (Table 3.8). Though the absorptivity of the compound was high at 212nm, it was not selected as the analytical wavelength due to its proximity to the lower limit of the UV transparent region of methanol (≈220nm). The absorbance was not measured at 236nm as the other materials used in the formulation of liposomes also having the absorption at same wavelength. The absorptivity at 274nm (13070 l/mol/cm, Table 3.8) was also found to be satisfactory and hence was selected as the analytical wavelength and used for further investigations. The regression equation was found to be Y = 0.021X –0.008.

A correlation coefficient of 0.9991 (Table 3.7) indicated that absorbance and concentration of the drug were linearly related. Beer's law was found to be obeyed between 5 and 50μg/ml (Table 3.8). Experimental and calculated values for the method are presented in Table 3.7. The slope of the regressed line (0.021, Table 3.7) indicates moderate sensitivity of the method as also seen by the value of the Sandell's sensitivity coefficient (4.3×10^{-5}), which, once again, reflects the moderate absorptivity of the compound.

There was a little variability between the experimental and regressed values. This conclusion was based on the low value of the variance of the response variable, S^2_{yx} (1.50×10^{-4}) and the low values of the standard error of the mean absorbances of the solutions used for preparing the calibration curve. The variance of the slope, S^2_b, was calculated as 9.52×10^{-8} whereas the intercept S^2_a, was determined to be 8.8×10^{-5}. The blank (methanol) does not interfere in the measurements as concluded from 't' test of the intercept (calculated 't' = 0.8528, tabulated 't' = 2.78 at 4 degrees of freedom, at 5% level).

The mean absorbance values of the methanolic solutions of tamoxifen citrate at different concentrations at preselected time intervals was determined, ANOVA studies of the results indicated no significant difference between the readings. Thus, tamoxifen citrate is stable over a period of 24h in methanol.

The presence of the other constituents of the liposomes such as phosphatidyl choline and cholesterol at the levels at which these materials were included in the liposomes, did not interfere with the estimation of tamoxifen citrate.

The method was used to estimate the amount of tamoxifen citrate entrapped in liposomes and its concentration in the supernatant, obtained during the recovery of the liposomes by centrifugation. Good mass balance was obtained between the amount of tamoxifen citrate added and that recovered from the liposomes and supernatant, signifying the suitability of the method for this application.

3.3.5 ESTIMATION OF CYTARABINE IN LIPOSOMES

Cytarabine in methanol yields a characteristic curve when scanned in the U.V. wavelength range between 200 to 400nm. The scan (Figure 3.3) shows absorption maxima at 209 and 274nm (Table 3.10). Though the absorptivity of the compound was high at 209nm, it was not selected as the analytical wavelength due to its proximity to the lower limit of the UV transparent region of methanol ($\simeq$ 220nm). The absorptivity at 274nm (8829 l/mol/cm, Table 3.10) was also found to be satisfactory and hence was selected as the analytical wavelength and used for further investigations. The regression equation was found to be $Y = 0.0355X + 0.015$.

A correlation coefficient of 0.9998 (Table 3.9) indicated that absorbance and concentration of the drug were linearly related. Beer's law was found to be obeyed between 5 and 50μg/ml (Table 3.10). Experimental and calculated values for the method are presented in Table 3.9. The slope of the regressed line (0.0355, Table 3.9) indicates moderate sensitivity of the method as also seen by the value of the Sandell's sensitivity coefficient (2.75×10^{-5}), which, once again, reflects the moderate absorptivity of the compound.

There was a little variability between the experimental and regressed values. This conclusion was based on the low value of the variance of the response variable, S^2_{yx} (2.6×10^{-4}) and the low values of the standard error of the mean absorbances of the solutions used for preparing the calibration curve.

The variance of the slope, S^2_b, was calculated as 1.70 x 10^{-7} whereas the intercept S^2_a, was determined to be 1.56 x 10^{-4}. The blank (methanol) does not interfere in the measurements as concluded from t' test of the intercept (calculated t' = 0.48, tabulated t' = 2.78 at 4 degrees of freedom at 5% level).

The mean absorbance values of the methanolic solutions of cytarabine at different concentrations at preselected time intervals was determined, ANOVA studies of the results indicated no significant difference between the readings. Thus, cytarabine is stable over a period of 24h in methanol.

The presence of the other constituents of the liposomes such as phosphatidyl choline and cholesterol at the levels at which these materials were included in the liposomes, did not interfere with the estimation of cytarabine.

In order to determine the precision and accuracy of the methods, known amounts of pure drug were subjected to recovery studies, using both the methods, in triplicate. Table 3.11 summarizes the results of these investigations. Accuracy of the method was ascertained by using the t' test at each level. The computed t' values of 25, 50 and 100mg were 0.50, 0.64 and 1.32 respectively. These values are lower than the tabulated t' value of 4.30 ($P \leq 0.05$) indicating no significant difference between the added and the estimated quantity. The precision of the method was evaluated using the S.D. of the results, the coefficient of variation and the relative mean error. The S.D, relative mean error and the coefficient of variation were found to be very low, which signifies the existence of good precision of the method.

The method was used to estimate the amount of cytarabine entrapped in liposomes and its concentration in the supernatant, obtained during the recovery of the liposomes by centrifugation. Good mass balance was obtained between the amount of cytarabine added and that recovered from the liposomes and supernatant, signifying the suitability of the method for this application.

3.3.6 ESTIMATION OF METHOTREXATE IN PHOSPHATE BUFFERED SALINE, pH 7.4 (PBS)

Methotrexate in phosphate buffered saline, pH 7.4 (PBS) yields a characteristic curve when scanned in the UV wavelength range between 200nm to 330nm with the scan (Figure 3.4) showing maxima at 303nm. The high absorptivity of the compound at 303nm (25182 l/mol/cm, Table 3.13) led the wavelength being chosen as the analytical wavelength. Linearity of the method was observed from a correlation coefficient of 0.9972 (Table 3.12). Beer's law was found to be obeyed between 5 – 50μg/ml (Table 3.13). Regression analysis of the experimental data was performed and is tabulated along with the experimental data in Table 3.12. The regression equation obtained was Y = 0.0517 X + 0.0274.

The variance of the response variable, S^2_{yx} was calculated as 6.33 x 10^{-4}. This low value illustrated the good fit between the determined and calculated data and thus indicated the low variability of the experiments. This low variability is also reflected in the low value of the standard error of the mean absorbances of the solutions used for obtaining the calibration curve. The variance of the slope, S^2_b was calculated as 2.53 x 10^{-6}. The value of the slope (0.0517, Table 3.12) indicates moderate sensitivity of the method and supported by the low value of the Sandell's sensitivity coefficient (6.4 x 10^{-5} μg/cm^2/0.001 abs unit, Table 3.13). The variance of the intercept, S^2_a, was calculated as 7.79 x 10^{-4}. The intercept was subjected to 't' test for significance and was not found to be significantly different from zero (calculated 't' = 0.9817, tabulated 't' = 3.18 at 3 degrees of freedom, at 5% level). This means that the blank (PBS) does not interfere in the absorbance measurements.

The mean absorbance values of the methotrexate solution in PBS at different concentrations at preselected time intervals was determined, ANOVA studies of the results indicated no significant difference between the readings. Thus, methotrexate is stable over a period of 72h in PBS.

The presence of the other constituents of the liposomes such as phosphatidyl choline and cholesterol at the levels at which these materials were included in the liposomes, did not interfere with the estimation of methotrexate.

Finally, the method was used to obtain the release profile of methotrexate from conventional and sterically stabilized liposomes *in vitro* using an appropriate set up. The reproducible drug release profiles obtained indicated that the developed method was suitable for the desired application.

3.3.7 ESTIMATION OF TAMOXIFEN CITRATE IN PHOSPHATE BUFFERED SALINE, pH 7.4 (PBS)

Tamoxifen citrate in phosphate buffered saline, pH 7.4 (PBS) yields a characteristic curve when scanned in the UV wavelength range between 200nm to 330nm with the scan (Figure 3.5) showing maxima at 236 and 274nm. Though the absorptivity of the compound was high at 236nm, it was not selected as the analytical wavelength due to the fact that the other materials used in the formulation of liposomes also having the absorption at same wavelength. The absorptivity at 274nm (9096 l/mol/cm, Table 3.15) was also found to be satisfactory and hence was selected as the analytical wavelength and used for further investigations. The regression equation was found to be Y = 0.0165x –0.0002.

The variance of the response variable, S^2_{yx} was calculated as 6.33 x 10^{-4}. This low value illustrated the good fit between the determined and calculated data and thus indicated the low variability of the experiments. This low variability is also reflected in the low value of the standard error of the mean absorbances of the solutions used for obtaining the calibration curve. The variance of the slope, S^2_b was calculated as 5.39 x 10^{-8}. The value of the slope (0.0165, Table 3.14) indicates moderate sensitivity of the method and supported by the low value of the Sandell's sensitivity coefficient (6.2 x 10^{-5} µg/cm²/0.001 abs unit, Table 3.13). The variance of the intercept, S^2_a, was calculated as 5.01 x 10^{-5}. The intercept was subjected to 't' test for significance and was not found to be significantly different from zero (calculated 't' = 0.028, tabulated 't' = 2.78 at 4 degrees of freedom, at 5% level). This means that the blank (PBS) does not interfere in the absorbance measurements.

The mean absorbance values of the methotrexate solution in PBS at different concentrations at preselected time intervals was determined, ANOVA studies of the results indicated no significant difference between the readings. Thus, methotrexate is stable over a period of 72h in PBS.

The presence of the other constituents of the liposomes such as phosphatidyl choline and cholesterol at the levels at which these materials were included in the liposomes, did not interfere with the estimation of tamoxifen citrate.

The method was used to obtain the release profile of tamoxifen citrate from conventional and sterically stabilized liposomes *in vitro* using an appropriate set up. The reproducible drug release profiles obtained indicated that the developed method was suitable for the desired application.

3.3.8 ESTIMATION OF CYTARABINE IN PHOSPHATE BUFFERED SALINE, pH 7.4 (PBS)

Cytarabine in phosphate buffered saline pH 7.4 (PBS) yields a characteristic curve when scanned in the U.V. wavelength range between 200 to 400nm. The scan (Figure 3.6) shows absorption maxima at 272nm, which was selected as the analytical wavelength for measuring the cytarabine in PBS. The absorptivity at 272nm (9683 l/mol/cm, Table 3.17) was also found to be satisfactory and hence was selected as the analytical wavelength and used for further investigations. The regression equation was found to be Y = 0.0395x - 0.0034.

A correlation coefficient of 0.9997 (Table 3.16) indicated that absorbance and concentration of the drug were linearly related. Beer's law was found to be obeyed between 5 and 50μg/ml (Table 3.17). Experimental and calculated values for the method are presented in Table 3.9. The slope of the regressed line (0.0395, Table 3.16) indicates moderate sensitivity of the method as also seen by the value of the Sandell's sensitivity coefficient (2.5 x 10^{-5}), which, once again, reflects the moderate absorptivity of the compound.

There was a little variability between the experimental and regressed values. This conclusion was based on the low value of the variance of the response variable, S^2_{yx} (3.9×10^{-4}) and the low values of the standard error of the mean absorbances of the solutions used for preparing the calibration curve. The variance of the slope, S^2_b, was calculated as 2.56×10^{-7} whereas the intercept S^2_a, was determined to be 2.36×10^{-4}. The blank (methanol) does not interfere in the measurements as concluded from t' test of the intercept (calculated $t' = 0.2214$, tabulated $t' = 2.78$ at 4 degrees of freedom, at 5% level).

The mean absorbance values of the cytarabine solution in PBS at different concentrations at preselected time intervals was determined, ANOVA studies of the results indicated no significant difference between the readings. Thus, cytarabine is stable over a period of 72h in PBS.

The presence of the other constituents of the liposomes such as phosphatidyl choline and cholesterol at the levels at which these materials were included in the liposomes, did not interfere with the estimation of cytarabine.

In order to determine the precision and accuracy of the methods, known amounts of pure drug were subjected to recovery studies, using both the methods, in triplicate. Table 3.18 summarizes the results of these investigations. Accuracy of the method was ascertained by using the t' test at each level. The computed t' values of 25, 50 and 100 mg were 0.46, 1.94 and 0.25 respectively. These values are lower than the tabulated t' value of 4.30 ($P \leq 0.05$) indicating no significant difference between the added and the estimated quantity. The precision of the method was evaluated using the S.D. of the results, the coefficient of variation and the relative mean error. The S.D, relative mean error and the coefficient of variation were found to be very low, which signifies the existence of good precision of the method.

3.3.9 ESTIMATION OF POLYETHYLENE GLYCOL DERIVATIVES IN LIPOSOMES

This assay method for the estimation of polyethylene glycol is based on complex formation between picrates and polyethylene glycols in dichloromethane. The complex in dichloromethane exhibits maximum absorbance at 378nm.

3.3.9.1 ESTIMATION OF METHOXY POLYETHYLENE GLYCOL 5000 ACTIVATED WITH CYANURIC CHLORIDE - PHOSPHATIDYL ETHANOLAMINE CONJUGATE (MPEG5000-CC-PE)

Absorptivity of the complex was calculated as 75639 l/mol/cm (Table 3.20). A correlation coefficient of 0.9982 (Table 3.19) indicated a linear relationship between absorbance and concentration of mPEG5000-CC-PE taken for complex formation. Beer's law was found to be obeyed between 10 – 100μg/ml (Table 3.20). The data of regression analysis on the collected data along with the raw data are presented in Table 3.19. The regression equation obtained was Y = 0.0126X + 0.0347

The variance of the response variable, S^2_{yx} was obtained as 4.32 x 10^{-4}. This low value denoted the closeness of experimental points to the least squares line and thus, the low variability in the procedure. This low variability can also be seen from the low value of the standard error of the mean absorbances of the solutions used for obtaining the calibration curve (Table 3.19). The variance of the slope, S^2_b was calculated as 7.1 x 10^{-8}. The value of the slope (0.0347, Table 3.19) indicated moderate sensitivity of the method, a fact supported by the value of the Sandell's sensitivity coefficient (7.68 x 10^{-5} μg/cm^2/0.001 abs unit, Table 3.20). The variance of the intercept, S^2_a, calculated was 2.6 x 10^{-4}. The significance of the intercept was examined using the null hypothesis.

The value of 't' was obtained as 2.15 whereas the value of 't' required for significance is 2.78 at 4 degrees of freedom at the 5% level. Thus, the null hypothesis can be accepted which means that the blank does not interfere in the absorbance measurements. The stability of the complex was ascertained by measurement of absorbances of the solutions, used for preparing the calibration curve, at regular intervals of time. It was observed that the colour of the complex was retained at its original intensity for an hour. Consequently, it is recommended that the absorbances be measured within an hour of sample preparation and this precaution was followed in all studies involving in this method.

The selectivity of the method for mPEG5000-CC-PE was established by estimating in the presence of the other major constituents of the liposomes viz. cholesterol, phosphatidyl choline, methotrexate, tamoxifen citrate and cytarabine at the levels at which these materials were included in the liposomes. None of the materials interfered in any way in the estimation of mPEG5000-CC-PE.

The method was used for estimating mPEG5000-CC-PE from liposomes and from the supernatant obtained in the centrifugation step used for removing the unentrapped drug. Mass balance studies revealed good correlation between the amount of mPEG5000-CC-PE added and the amounts recovered from the liposomes and supernatant. Thus the method was found to be satisfactory for estimation of mPEG5000-CC-PE in liposomes.

3.3.9.2 ESTIMATION OF METHOXY POLYETHYLENE GLYCOL 2000 ACTIVATED WITH CYANURIC CHLORIDE - PHOSPHATIDYL ETHANOLAMINE CONJUGATE (MPEG2000-CC-PE)

Absorptivity of the complex was calculated as 32677 l/mol/cm (Table 3.22). A correlation coefficient of 0.9988 (Table 3.21) indicated a linear relationship between absorbance and concentration of mPEG2000-CC-PE taken for complex formation. Beer's law was found to be obeyed between 10 – 100μg/ml (Table 3.22). The data of regression analysis on the collected data along with the raw data are presented in Table 3.21. The regression equation obtained was Y = 0.0115X + 0.0034.

The variance of the response variable, S^2_{yx} was obtained as 2.49×10^{-4}. This low value denoted the closeness of experimental points to the least squares line and thus, the low variability in the procedure. This low variability can also be seen from the low value of the standard error of the mean absorbances of the solutions used for obtaining the calibration curve (Table 3.21). The variance of the slope, S^2_b was calculated as 4.09×10^{-8}. The value of the slope (0.0115, Table 3.21) indicated moderate sensitivity of the method, a fact supported by the value of the Sandell's sensitivity coefficient (8.6×10^{-5} μg/cm^2/0.001 abs unit, Table 3.22). The variance of the intercept, S^2_a, calculated was 1.51×10^{-4}. The significance of the intercept was examined using the null hypothesis.

The value of t' was obtained as 0.276 whereas the value of t' required for significance is 2.78 at 4 degrees of freedom at the 5% level. Thus, the null hypothesis can be accepted which means that the blank does not interfere in the absorbance measurements. The stability of the complex was ascertained by measurement of absorbances of the solutions, used for preparing the calibration curve, at regular intervals of time. It was observed that the colour of the complex was retained at its original intensity for an hour. The selectivity of the method for mPEG2000-CC-PE was established by estimating in the presence of the other major constituents of the liposomes viz. cholesterol, phosphatidyl choline, methotrexate, tamoxifen citrate and cytarabine at the levels at which these materials were included in the liposomes. None of the materials interfered in any way in the estimation of mPEG2000-CC-PE.

The method was used for estimating mPEG2000-CC-PE from liposomes and from the supernatant obtained in the centrifugation step used for removing the unentrapped drug. Mass balance studies revealed good correlation between the amount of mPEG2000-CC-PE added and the amounts recovered from the liposomes and supernatant. Thus the method was found to be satisfactory for estimation of mPEG2000-CC-PE in liposomes.

3.3.9.3 ESTIMATION OF METHOXY POLYETHYLENE GLYCOL 5000 ACTIVATED WITH IMIDAZOLINE CARBONYL - PHOSPHATIDYL ETHANOLAMINE CONJUGATE (MPEG5000-IC-PE)

Absorptivity of the complex was calculated as 79352 l/mol/cm (Table 3.24). A correlation coefficient of 0.9981 (Table 3.23) indicated a linear relationship between absorbance and concentration of mPEG5000-IC-PE taken for complex formation. Beer's law was found to be obeyed between 10 – 100μg/ml (Table 3.24). The data of regression analysis on the collected data along with the raw data are presented in Table 3.23. The regression equation obtained was Y = 0.0131X + 0.036.

The variance of the response variable, S^2_{yx} was obtained as 4.93 x 10^{-4}. This low value denoted the closeness of experimental points to the least squares line and thus, the low variability in the procedure. This low-variability can also be seen from the low value of the standard error of the mean absorbances of the solutions used for obtaining the calibration curve (Table 3.23). The variance of the slope, S^2_b was calculated as 8.1 x 10^{-8}. The value of the slope (0.036, Table 3.23) indicated moderate sensitivity of the method, a fact supported by the value of the Sandell's sensitivity coefficient (7.3 x 10^{-5} μg/cm^2/0.001 abs unit, Table 3.24). The variance of the intercept, S^2_a, calculated was 2.98 x 10^{-4}. The significance of the intercept was examined using the null hypothesis.

The value of 't' was obtained as 2.09 whereas the value of 't' required for significance is 2.78 at 4 degrees of freedom at the 5% level. Thus, the null hypothesis can be accepted which means that the blank does not interfere in the absorbance measurements. The stability of the complex was ascertained by measurement of absorbances of the solutions, used for preparing the calibration curve, at regular intervals of time. It was observed that the colour of the complex was retained at its original intensity for an hour.

The selectivity of the method for mPEG5000-IC-PE was established by estimating in the presence of the other major constituents of the liposomes viz. cholesterol, phosphatidyl choline, methotrexate, tamoxifen citrate and cytarabine at the levels at which these materials were included in the liposomes. None of the materials interfered in any way in the estimation of mPEG5000-IC-PE.

The method was used for estimating mPEG5000-IC-PE from liposomes and from the supernatant obtained in the centrifugation step used for removing the unentrapped drug. Mass balance studies revealed good correlation between the amount of mPEG5000-IC-PE added and the amounts recovered from the liposomes and supernatant. Thus the method was found to be satisfactory for estimation of mPEG5000-IC-PE in liposomes.

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